PRETREATMENT OF ISOLATED HUMAN PERIPHERAL BLOOD LYMPHOCYTES WITH L-OXOTHIAZOLIDINE 4-CARBOXYLATE REDUCES SULFUR MUSTARD CYTOTOXICITY

Clark L. Gross and William J. Smith

Biochemical Pharmacology Branch
USAMRICD, APG, MD 21010-5425

ABSTRACT

Despite 70 years of research, there appears to be no satisfactory prophylaxis or treatment for the vesicant chemical warfare agent sulfur mustard (HD). Attempts to modify cytotoxicity of HD are now focusing on the use of intracellular "scavengers" to interact with sulfur mustard before it can react with critical targets within the cell. Glutathione (GSH) is known to react readily with HD and is involved in the major metabolic pathway to HD detoxification. Glutathione level within the cell was raised 40-60% over control values by pretreatment of quiescent human peripheral blood lymphocytes (PBL) with 10 mM L-oxothiazolidine-4-carboxylate (OTC), a "masked" cysteine precursor. This increase in glutathione level was not toxic to the cells as judged by trypan blue dye exclusion and reached a maximum level in 48 hrs. PBL pretreated with 10 mM OTC for 48 hrs were harvested, washed, and exposed to 10, 50, or 100 uM HD. After an additional 48 hrs of incubation at 37°C, cytotoxicity was measured by propidium iodide dye uptake using flow cytometry. Pretreatment with OTC led to a 20% decrease in cytotoxicity with 10 uM HD, an 11% decrease in cytotoxicity with 50 uM HD, and an 8% decrease in cytotoxicity with 100 uM HD. Cytotoxicity of HD was not influenced by addition of 10 mM OTC 2 hrs after HD exposure. These results suggest that biochemical manipulation of intracellular GSH level may provide an important pretreatment regimen to reduce the cytotoxicity of HD.

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INTRODUCTION

Sulfur mustard (2,2'-dichlorodiethyl sulfide, HD) is a potent vesicant compound that causes serious injury to the eyes, lung, and skin. Although a biochemical hypothesis has been published to explain its vesicant action (Papirmeister et al, 1985), the exact mechanism responsible for causing the injury remains obscure. No useful pretreatment or therapy against this compound is available despite some 70 years of research following its first use in WWI.

Sulfur mustard reacts extensively with glutathione when absorbed systemically (Davison et al., 1961; Black et al., 1992) and also reacts with other thiols (Walker et al., 1967). In addition, sulfur mustard seems to exert other effects at sites distant from the exposure site (Elsayed et al, 1989). Increasing glutathione concentrations within lymphocytes by using N-acetyl cysteine appears to have a beneficial effect in protecting cells against the cytotoxic action of HD (Gross et al., 1993). The use of other precursors of glutathione to stimulate its synthesis may also be helpful in protecting cells. L-Oxothiazolidine-4-carboxylate (OTC) is a "masked" cysteine precursor that is converted to cysteine by the enzyme, 5-oxoprolinase and leads to increased levels of glutathione within the liver (Williamson et al., 1979). This compound has also been administered to humans and was found to raise intracellular levels of both cysteine and glutathione in lymphocytes (Porta et al., 1991).

This study was undertaken to determine whether pretreatment of cells with this compound can also be used to protect cells against HD cytotoxicity. Human peripheral blood lymphocytes (PBL) were selected to be the model in this study since these cells are more sensitive than keratinocytes to the toxic effects of HD (Smith et al., 1990, 1992) and appear to be a suitable in vitro model for studying this vesicant agent (Meier et al, 1987, 1992; Gross et al., 1993).

METHODS

Isolation of human peripheral lymphocytes: Blood was obtained by venipuncture from volunteers using an approved human use protocol. Peripheral blood lymphocytes (PBL) were isolated as previously described (Meier et al., 1987). Initial cell counts and viability measurements were determined by exclusion of trypan blue dye and a hemacytometer.

Exposure of lymphocytes to OTC: Isolated PBL were resuspended in RPMI 1640 + gentamycin (50 ug/ml) and incubated in 24-well tissue culture plates. OTC at the appropriate concentration was added, and the plates were incubated for up to 72 hr at 37° C in a 5% CO_2 incubator. At the indicated time, the cells were harvested by centrifuging the plate at 450 g, 20°C, 20 min and supernatants were aspirated off. The plates were frozen on dry ice and then stored at -80° C until assayed for glutathione.

Exposure of pretreated lymphocytes to sulfur mustard: PBL were incubated in a 25 cm^2 flask for 48 hours at 37° C in a 5% CO_2 incubator with or without 10 mM OTC. Cells were harvested by aspiration, placed in a 50 ml conical tube, and centrifuged at 450g for 10 min at 20°C. The cell pellet was washed 2x with PBS, centrifuged as above, and resuspended in

RPMI 1640 + gentamycin (50 ug/ml). The cell concentration was adjusted to 1 x 10^7 cells/ml and cells were dispensed into 24-well tissue culture plates (3 x 10^6 cells/well). An aliquot of cells was also removed, centrifuged, and immediately frozen for analysis of initial glutathione level. HD was added to the plate in an approved fume hood. The plates were left in the hood for 2 hrs to allow complete hydrolysis of HD and then placed in the CO_2 incubator for an additional 46 hours before being harvested. Cytotoxicity measurements were performed by flow cytometric analysis as described previously (Smith et al., 1991).

Glutathione analysis: The frozen lymphocytes in the 24-well plates were disrupted by adding 150 ul of 5% 5-sulfosalicylic acid and the plate was mixed and placed at 4°C overnight. Contents were removed and centrifuged for 3 min at 8,800 g, and supernatants were assayed for glutathione (GSH + GSSG) by using the glutathione reductase cycling assay (Griffith, 1980). Absorbance measurements were made on a Beckman DU-70 spectrophotometer.

RESULTS

When lymphocytes were incubated with various concentrations of OTC for 24 hours, there was little increase in glutathione levels in cells incubated with OTC for 24 hours compared to untreated control cells. When the incubation time was extended to 48 hrs, there was an increase of about 40% in glutathione level with 10 mM OTC (Figure 1). OTC was not toxic at these concentrations since viabilities of the treated cells were comparable to untreated controls (data not shown).

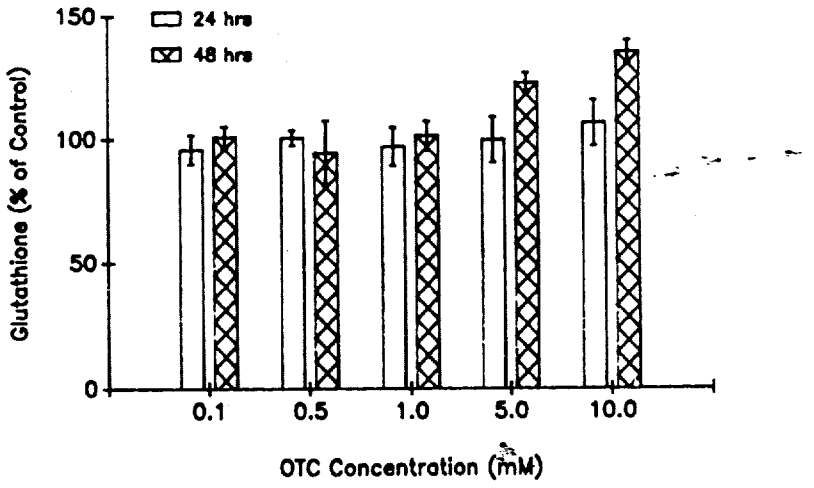

Figure 1. Effect of various [OTC] on glutathione levels in PBL over a 24- and 48- hr period. Glutathione levels were measured as described in METHODS. Each bar represents the mean ± 1 S.D. of 3 separate determinations.

Cells were incubated for an extended period (up to 72 hours) with 10 mM OTC to find an optimum time for pretreatment. Figure 2 again shows an increased level of glutathione within 48 hours (55%) followed by a decrease to control values by 72 hours. Incubation of PBL with 10 mM OTC for up to 72 hours did not reveal any cytotoxicity (data not shown).

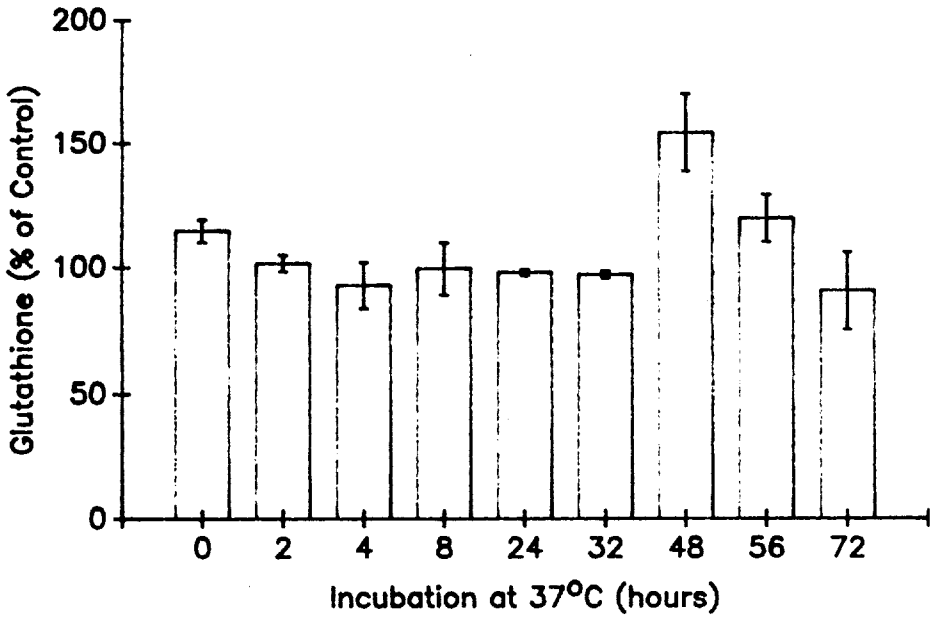

Figure 2. Cells were incubated for up to 72 hrs with 10 mM OTC. Cells were harvested at the indicated times and the glutathione levels were determined as described in METHODS. Each bar represents the mean ± 1 S.D. of 3 separate determinations.

Cells pretreated with or without 10 mM OTC for 48 hours were harvested from flasks and dispensed into 24-well plates. Aliquots of these cells were also removed for glutathione analysis. (OTC pretreated cells had a 60% higher glutathione level than control cells.) The cells were then exposed to 10, 50, or 100 uM HD and incubated for an additional 48 hours before they were harvested. Cytotoxicity was determined by propidium iodide and flow cytometric analysis. Figure 3 shows that OTC pretreatment led to a 20% decrease in cytotoxicity with 10 uM HD, an 11% decrease in cytotoxicity with 50 uM HD, and an 8% decrease in cytotoxicity with 100 uM HD after a 48 hour post-treatment incubation.

The therapeutic potential of OTC was also investigated by adding 10 mM OTC to cells 2 hours after they had been exposed to 10, 50, or 100 uM HD. Figure 4 shows the failure of this compound to protect against HD-induced cytotoxicity when administered as a treatment.

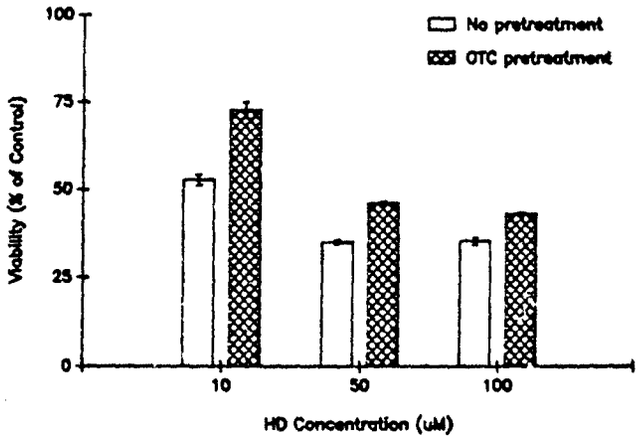

Figure 3. The effect of 10 mM OTC pretreatment on PBL that were exposed to various HD concentrations. PBL were incubated for an additional 48 hrs before cytotoxicity was measured by flow cytometry. Each bar represents the mean ± 1 S.D. of 3 separate determinations.

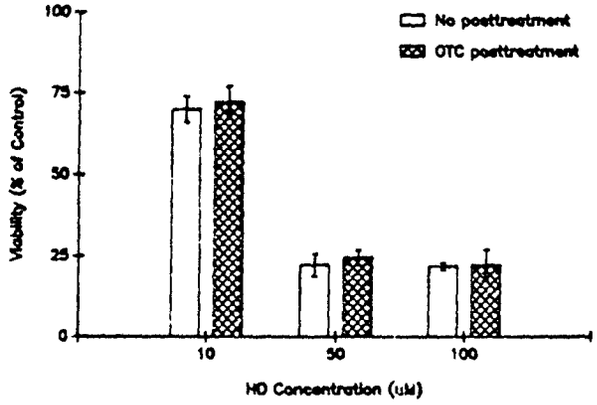

Figure 4. PBL were exposed to various HD concentrations for 2 hrs. 10 mM OTC was added after the HD had reacted and the PBL were incubated for an additional 48 hrs before cytotoxicity was measured by flow cytometry. Each bar represents the mean ± 1 S.D. of 3 separate determinations.

DISCUSSION

Increasing intracellular levels of glutathione within normal cells by biochemical manipulation may also grant some partial resistance to HD by reducing alkylation of critical cellular macromolecules. Glutathione could then serve as an alternative alkylating site or intracellular "scavenger" for HD. Glutathione levels in PBL were increased by pretreatment with N-acetyl-L-cysteine (NAC) and appeared to have some partial efficacy against HD (Gross et al., 1993). NAC increases glutathione levels in liver and is the drug of choice against poisoning by acetaminophen.

L-oxothiazolidine-4-carboxylate (OTC) is also used as an intracellular delivery system for cysteine and appears to be more effective than N-acetyl-L-cysteine against acetaminophen poisoning in mice (Williamson et al., 1982). Human volunteers given OTC orally show a significant increase of both cysteine and glutathione levels in lymphocytes as well as an increase in plasma cysteine 2-3 hours after ingestion (Porta et al., 1991). Increasing the intracellular levels of both cysteine and glutathione in cellular targets by OTC pretreatment may help in reducing tissue damage by HD.

These preliminary results show that pretreatment of cells with OTC can increase intracellular glutathione content 40-60% in PBL from different individual donors. Elevated glutathione levels appear to be partially protective against HD-induced cytotoxicity. The increase in intracellular glutathione level is presumably due to the conversion of OTC to cysteine by oxoprolinase (Williamson and Meister, 1981) and its subsequent incorporation into glutathione. Studies comparing NAC and OTC may prove useful in formulating a pretreatment regimen for HD intoxication.

REFERENCES

BLACK, R.M., BREWSTER, K., CLARKE, R.J., HAMBROOKE, J.L., HARRISON, J.M., and HOWELLS, D.J., (1992). Biological fate of sulphur mustard 1.1'-thiobis(2-chloroethane): isolation and identification of urinary metabolites following intraperitoneal administration to rat. Xenobiotica 22, 405-418.

DAVISON, C., ROZMAN, R.S., and SMITH, P.K. (1961). Metabolism of Bis-B-chloroethyl sulfide (sulfur mustard gas). Biochem. Pharmacol. 7, 64-74.

ELSAYED, N.B., OMAYE, S.T., KLAIN, G.J., INASE, J.L., DAHLBERG, E.T., WHEELER, C.R., and KORTE, D.W. (1989). Response of mouse brain to a single subcutaneous injection of the monofunctional sulfur mustard, butyl 2-chloroethyl sulfide (BCS). Toxicol. 58, 11-20.

GRIFFITH, O.W. (1980). Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. Anal. Biochem. 106, 207-212.

GROSS, C.L., INNACE, J.K., HOVATTER, R.C., MEIER, H.L., and SMITH, W.J., (1993) Biochemical manipulation of intracellular glutathione levels influences cytotoxicity to isolated human lymphocytes by sulfur mustard, Cell Biol. and Tox. (in press).

MEIER, H.L., GROSS, C.L., and PAPIRMEISTER, B. (1987). 2,2'-dichlorodiethyl sulfide decreases NAD+ levels in human leukocytes. Toxicol. Lett. 39, 109-122.

MEIER, H.L. and JOHNSON, B.J. (1992). The determination and prevention of cytotoxic effects induced in human lymphocytes by the alkylating agent 2,2' dichlorodiethyl sulfide (sulfur mustard, HD), Toxicol. Appl. Pharmacol. 113, 234-239.

MEISTER, A. and ANDERSON, M. E. (1983). Glutathione. Ann. Rev. Biochem. 52, 711-760.

MOSLEN, M.T., WHITEHEAD, R.F., FERGUSON, A.E., and KANZ, M.F. (1989). Protection by L-2-oxothiazolidine-4-carboxylate, a cysteine prodrug, against 1,1-dichloroethylene hepatotoxicity in rats is associated with decreases in toxin metabolism and cytochrome P-450. J. Pharmacol. Exp. Ther. 248, 157-163.

PAPIRMEISTER, B., GROSS, C.L., MEIER, H.L., PETRALI, J.P., and JOHNSON, J.B. (1985). Molecular basis for mustard-induced vesication. Fund. Appl. Toxicol. 5, S134-S149.

PORTA, P., AEBI, S., SUMMER, K., AND LAUTERBURG, B.H., (1991). L-2-Oxothiazolidine-4-carboxylic acid, a cysteine prodrug: pharmacokin- etics and effects on thiols in plasma and lymphocytes in human. J. Pharmacol. Exp. Ther. 257, 331-334.

SMITH, W.J., GROSS, C.L., CHAN, P., and MEIER, H.L. (1990). The use of human epidermal keratinocytes in culture as a model for studying the biochemical mechanisms of sulfur mustard toxicity. Cell Biol. Toxicol. 6, 285-291.

SMITH, W.J., SANDERS, K.M., GALES, Y.A., and GROSS, C.L. (1991). Flow cytometric analysis of toxicity by vesicating agents in human cells in vitro. J. Toxicol. - Cut. & Ocular Toxicol. 10, 33-42.

WALKER, I.G., and SMITH, J.F. (1969). Protection of L-cells by thiols against the toxicity of sulfur mustard. Can. J. Physiol. Pharmacol. 47, 143-151.

WILLIAMSON, J.M., and MEISTER, A. (1981). Stimulation of hepatic glutathione formation by administration of L-2-oxothiazolidine-4-carboxylate, a 5-oxo-L-prolinase substrate. Proc. Natl. Acad. Sci., 78 (2), 936-939.